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Motor-Circuit Communication Matrix from Spinal Cord to Brainstem Neurons Revealed by Developmental Origin

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SUMMARY

Accurate motor-task execution relies on continuous comparison of planned and performed actions. Motor-output pathways establish internal circuit collaterals for this purpose. Here we focus on motor collateral organization between spinal cord and upstream neurons in the brainstem. We used a newly developed mouse genetic tool intersectionally with viruses to uncover the connectivity rules of these ascending pathways by capturing the transient expression of neuronal subpopulation determinants. We reveal a widespread and diverse network of spinal dual-axon neurons, with coincident input to forelimb motor neurons and the lateral reticular nucleus (LRN) in the brainstem. Spinal information to the LRN is not segregated by motor pool or neurotransmitter identity. Instead, it is organized according to the developmental domain origin of the progenitor cells. Thus, excerpts of most spinal information destined for action are relayed to supraspinal centers through exquisitely organized ascending connectivity modules, enabling precise communication between command and execution centers of movement.

INTRODUCTION

Movement is the behavioral output of neuronal circuits computing motor commands and performance. The muscular system of the body acts according to instructions that supraspinal centers convey by descending pathways to the spinal cord, which in turn delivers these commands to muscles through motor neurons eliciting movement. The central nervous system (CNS) employs two circuit-level strategies to monitor planned and performed motor actions (Crapse and Sommer, 2008; Poulet and Hedwig, 2007; Sommer and Wurtz, 2008; Wolpert and Miall, 1996). First, motor-output pathways establish axon collaterals at many levels, providing internal efference copy signals of planned action to recipient neurons. Second, movement-evoked sensory feedback from the body reaches the CNS to report on performed motor actions. Together, these two distinct information streams are used to adjust and modify descending motor commands accordingly. Despite their undisputed role in influencing motor behavior, surprisingly little is known about identity, composition, and synaptic organization of core circuit elements encompassing these pathways, undoubtedly fundamental information needed to understand their function.

Efference copy pathways arising from spinal neurons with direct connections to motor neurons represent a suitable entry point to address these challenging questions. Landmark studies in the cat by Orlovsky and collaborators demonstrated that locomotor-related ascending signaling streams from the spinal cord are transmitted to supraspinal centers by two main pathways (for review, see Arshavsky et al., 1986; Orlovsky et al., 1999). Of highest relevance to this study, the lateral reticular nucleus (LRN) in the caudal medulla receives synaptic input strongly correlated with ongoing spinal intrinsic information in a manner independent of peripheral sensory feedback, and its activity is in turn relayed to the cerebellum by mossy fibers (Arshavsky et al., 1978, 1986; Brodal, 1949; Orlovsky et al., 1999; Oscarsson, 1965). Pharmacological ablation of the LRN in the cat leads to movement deficits related to postural balance and paw placement (Santarcangelo et al., 1981). A spinal subsystem projecting to the LRN comprised of neurons referred to as C3-C4 propriospinal neurons (PNs) is implicated in voluntary forelimb motor control in cat and monkey (for review, see Alstermark and Isa, 2012; Alstermark et al., 2007). C3-C4 PNs are located at cervical levels C3 to C4 and have the special feature of bifurcating axonal projections, with one ascending branch to the LRN and a second descending branch establishing direct synaptic connections to cervical motor neurons at C5 to C8 spinal levels. Collaterals of C3-C4 PNs hence transmit an efference copy signal of premotor information directly to the LRN, leading to coincident regulation of motor neurons and LRN neurons. However, whether this neuronal system is constructed as a single homogeneous reporting channel or monitors motor-output pathways more generally through functionally diverse neuronal subpopulations is currently entirely unknown and represents a conceptually important question to address.

Recent work in the mouse has provided a wealth of information about genetic specification of neuronal subpopulations and their function in the spinal cord (reviewed by Alaynick

et al., 2011; Arber, 2012; Goulding, 2009; Grillner and Jessell, 2009; Kiehn, 2011). These studies demonstrate that spinal neurons can be subdivided into 11 cardinal classes based on their progenitor-domain origin (dorsal: dI1–dI6; ventral: V0–V3; MN: spinal motor neurons), and genetic mutation or silencing experiments reveal a variety of distinct roles of corresponding neuronal subpopulations (reviewed by Arber, 2012; Goulding, 2009; Grillner and Jessell, 2009; Kiehn, 2011). In addition, transsynaptic virus tools (Wickersham et al., 2007) determined the distribution of spinal interneurons with monosynaptic connections to individual motor neuron pools, revealing broad segmental but highly stereotypic patterns (Stepien et al., 2010; Tripodi et al., 2011). The availability of genetic entry points to virtually any spinal neuron in the mouse and the possibility to visualize neurons with direct motoneuronal connections have opened the door to determining functional diversity and connectivity profiles of ascending spinal populations to the brainstem.

Here we unravel the connectivity profiles of ascending pathways from the spinal cord to supraspinal centers, taking intersectional approaches between mouse genetic and viral tools. We find that bifurcating premotor PNs with collaterals to the LRN surprisingly represent a set of highly diverse neuronal populations with residence throughout the cervical and rostral thoracic spinal cord. Neuronal diversification is uncovered by differential genetic identity based on progenitor-domain origin during development, which foreshadows distinct neuronal settling positions in the spinal cord. Moreover, genetically diverse spinal subpopulations establish a highly selective and organized connection map to the LRN. Our findings support a model in which the LRN represents a major hub for selective combination of functionally diverse ascending spinal information in order to extract an excerpt of most ongoing motor activity needed for execution of motor tasks.

RESULTS

FL-Premotor Axons Terminate in Ipsilateral LRN

To visualize brainstem areas targeted by efference copy information of neurons premotor to limb-innervating motor neurons, we used transsynaptic rabies technology with monosynaptic restriction (Stepien et al., 2010; Tripodi et al., 2011; Wickersham et al., 2007). These premotor neurons are defined by their monosynaptic connections to lateral motor column (LMC) motor neurons, innervating either forelimb (FL; LMC^{FL}; Figure 1A) or hindlimb (HL; LMC^{HL}) muscles. We targeted combined injection of glycoprotein-deficient rabies-mCherry virus and adeno-associated virus (AAV) expressing glycoprotein broadly into different FL or HL muscles to retrogradely infect and initiate transsynaptic spread from LMC^{FL} or LMC^{HL} motor neurons, respectively (Figure 1A).

In limb-muscle injections with monosynaptic rabies viruses, analysis revealed strong and selective targeting of the LRN by FL- but not HL-premotor axons. Notably, the termination site was exclusively in the LRN ipsilaterally to the injected FL muscles (Figures 1A and 1B). We identified the LRN by its location at caudal brainstem levels in a position ventral to the Ambiguus motor nucleus ($ChAT^{ON}$) (Paxinos and Franklin, 2012). Moreover, we found it to be surrounded by but entirely devoid of glycinergic neurons (Figure 1B; visualized in $GlyT2^{eGFP}$ mice).

In addition to the LRN, we found that FL-premotor axons also terminate in most cranial motor nuclei both ipsi- and contralaterally to the injected limb (Figure S1A available online). FL-premotor axons also projected to the external cuneate nucleus (ECN) located dorsally at caudal brainstem levels. The ECN is known for its inputs from dorsal root ganglia (DRG) sensory neurons and in turn projects to the cerebellum through mossy fibers (Campbell et al., 1974; Oscarsson, 1965; Rosén, 1969) (Figure 1C). In contrast, the more rostrally located pontine precerebellar nucleus also giving rise to cerebellum-projecting mossy fibers was devoid of FL-premotor input (Figure 1D).

To determine whether FL-premotor axons target the entire LRN, we next combined monosynaptically restricted transsynaptic marking of FL-premotor axons with cerebellar injections to retrogradely label LRN neurons (Figure 1E). We found that FL-premotor axons only target a specific region of the caudal LRN with high axonal density, located in a central but dorsally restricted area (Figures 1F–1H). To delineate whether LRN axonal-targeting specificity is related to motor neuron pool identity from which the rabies spread is initiated, we injected different FL muscles to label corresponding premotor neurons. We found that premotor axons terminate broadly within the previously mapped FL-premotor LRN territory irrespective of muscular identity for four FL muscle groups (Figure 1I). By contrast and as previously described in the cat (Alstermark et al., 1985, 1991), neck premotor neurons failed to terminate within the LRN (Figure 1I). Analysis of motor-pool-specific premotor input to ECN revealed more segregated input specificity (Figure S1B), consistent with previous electrophysiological studies (Campbell et al., 1974; Rosén, 1969).

Together, these findings demonstrate that although premotor input to brainstem structures is highly specific, FL-premotor neurons associated with individual functionally distinct motor neuron pools do not exhibit profound input specificity within the LRN premotor FL-targeted area.

Spinal Cord Provides Prominent Source of Synaptic Input to LRN

To delineate possible sites of cellular origin that contribute to synaptic input to the LRN, we used a triple-virus-injection approach to initiate monosynaptic transsynaptic spread selectively from LRN neurons. We first targeted LRN neurons from the cerebellum by Cav-Cre injection, followed by sequential intra-LRN injection with AAV-flex-TVA/G and EnvA-coated rabies-mCherry (Figure 2A). This approach led to high targeting specificity of LRN neurons at high efficiency (Figure 2B).

We found many neurons marked throughout the spinal cord and at cervical levels, with the majority of LRN connecting neurons residing ipsilaterally to injection, whereas contralateral neurons were confined to Rexed's lamina 8 (Figure 2C). Supraspinally, we observed only few additional structures labeled dominantly using this approach. Most notably, the contralateral red nucleus contained many labeled neurons, in agreement with previous studies (Hinman and Carpenter, 1959; Walberg, 1958). We also noted a distinct cluster of neurons in the rostral ventral respiratory group (rVRG) dorsal to the contralateral LRN

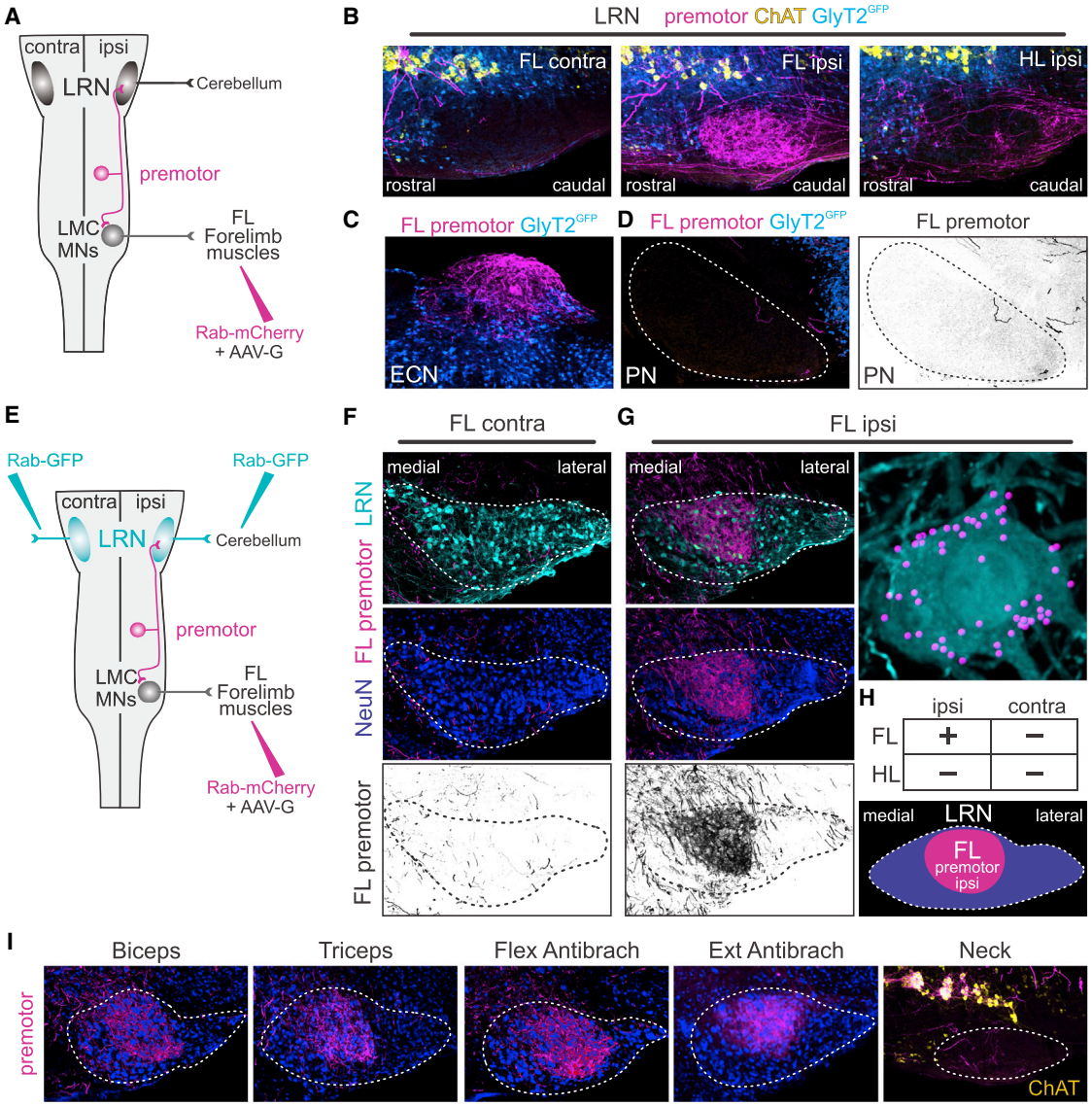

Figure 1. Forelimb Premotor Axons Terminate in Ipsilateral LRN

(A) Scheme of experimental setup displaying FL-premotor neurons labeled by monosynaptic retrograde spreading from FL LMC motor neurons upon coinjection of FL muscles with Rab-mCherry and AAV-G protein. Assay is used to determine presence of axonal terminals of premotor neurons in contra- and ipsilateral LRN.

(B) Analysis of contra- and ipsilateral LRN on sagittal sections upon broad FL or HL muscle injection of monosynaptic rabies virus in *GlyT2*^{GFP} mice. FL-premotor terminals target ipsi- but not contralateral LRN, in an area ventral to ChAT^{on} Ambiguus motor neurons and devoid of glycinergic neurons.

(C and D) FL-premotor input to ipsilateral external cuneate nucleus (ECN) and pontine nucleus (PN) on sagittal sections upon monosynaptic rabies injections into *GlyT2*^{GFP} mice.

(E) Scheme of experimental setup as in (A) but with additional bilateral retrograde infection of LRN neurons from the cerebellum.

(F and G) Retrogradely marked LRN neurons, FL-premotor terminals, and NeuN contralateral (F) and ipsilateral (G) to FL muscle injections (coronal sections, midway along rostro-caudal LRN dimension). High-resolution panel in (G) to the right depicts apposition of direct premotor input (purple) to LRN neurons on the side ipsilateral to FL injection.

(H) Summary diagram depicting confined termination of FL-premotor neuron input to central core domain of LRN ipsilateral to limb-muscle injection (table on top: [+] indicates input; [–] indicates no input by corresponding premotor axons to LRN).

(I) FL-premotor input to ipsilateral LRN upon monosynaptic rabies injections into biceps, triceps, flexor antibrachium, extensor antibrachium, and neck muscles. See also Figure S1.

and in close proximity to ChAT^{ON} Ambiguus motor neurons (Figure 2D) (Ezure and Tanaka, 1997). In agreement with these findings, we also found that contralateral phrenic motor neurons in the spinal cord received highly selective synaptic input by pre-LRN rabies marked axons (Figure 2D). Motor cortex provided minor input to the LRN, with a low density of terminals mainly surrounding the LRN (Figure S2), consistent with previous experiments in the rat (Rajakumar et al., 1992). Together, these data

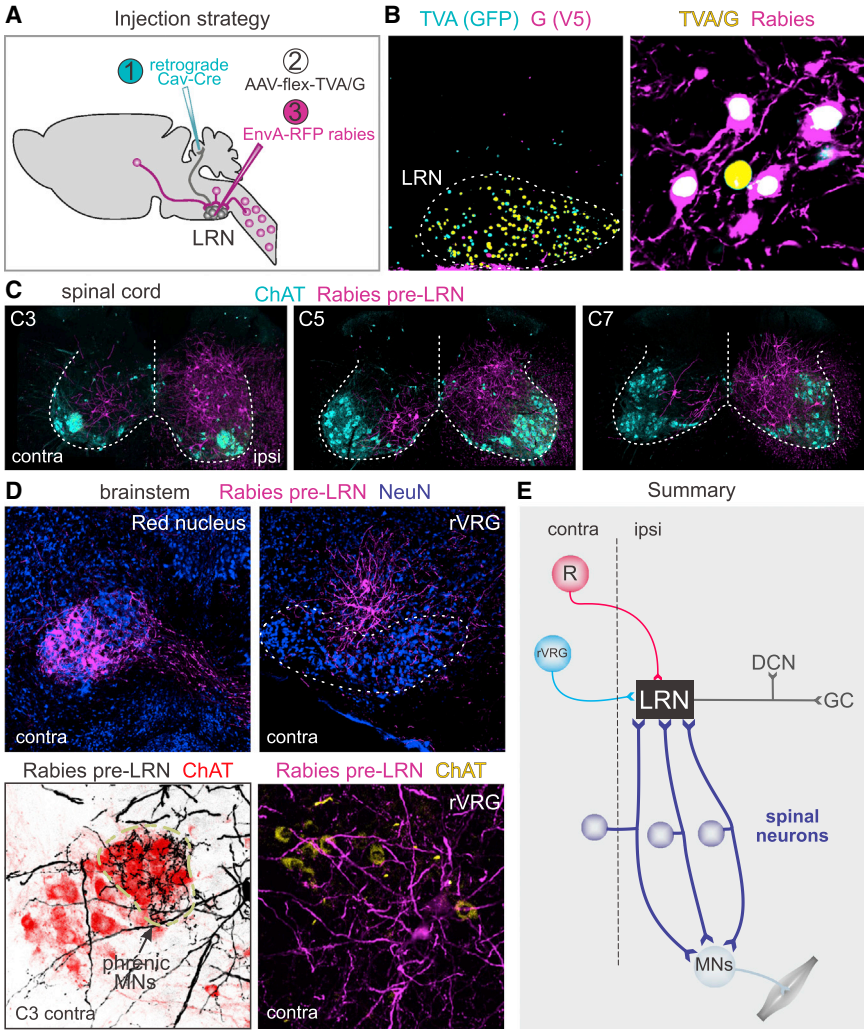

Figure 2. Synaptic Input to LRN Revealed by Transsynaptic Rabies

(A) Scheme for sequential injections to initiate monosynaptic transsynaptic rabies spread from LRN neurons.

(B) Coronal LRN section analysis to determine infection (left: low-resolution overview depicting high coinfection rate of TVA and G viruses; right: triple-infected neurons).

(C) Pre-LRN neurons on spinal cord sections are located both ipsi- and contralaterally to injection.

(D) Supraspinal labeled pre-LRN populations include neurons in the red nucleus (R) and rostral ventral respiratory group (rVRG). Input of pre-LRN axons to phrenic motor neurons (bottom left) in the contralateral spinal cord and intermingling of pre-LRN neurons with ChAT^{ON} Ambiguus motor neurons (bottom right) confirm rVRG identity.

(E) Summary of synaptic input to the LRN, depicting major input sources from the spinal cord, as well as supraspinal input from R and rVRG.

See also Figure S2.

demonstrate that LRN neurons receive prominent synaptic input from spinal neurons (Figure 2E), thus directing our further analysis to the spinal cord.

Broad Spinal Residence of Neurons Providing FL-Premotor Input to LRN

To identify the source of premotor axons in the LRN, we combined retrograde axonal infection from the LRN and transsynaptic marking of premotor neurons by FL muscle injections with monosynaptic rabies tools (Figure 3A). Although such a strategy is not suitable to quantify the absolute number of dually connecting neurons due to the limited time window during which two different rabies viruses can coinfect the same neuron (Ugolini, 2010), it has been used successfully before to visualize the overall distribution of dual-connection neurons (Stepien et al., 2010). We found that at cervical levels, the distribution of spinal neurons projecting to the LRN was similar to that of FL-premotor neurons (Stepien et al., 2010), with a majority of neurons located ipsilaterally and with contralateral neurons mainly confined to Rexed's lamina 8 (Figure S3). The distribution patterns of spinal neurons projecting to the LRN morphed gradually into one with a contralateral dominance at lumbar levels (Figure S3), in agreement with previous studies (Koekkoek and Ruigrok, 1995; Shokunbi et al., 1985).

We next mapped the three-dimensional distribution of dually rabies-labeled neurons in the spinal cord, projecting to the LRN and connecting to FL-motor neurons (Figure 3A). We found that such neurons were distributed broadly over cervical spinal segments, with double-rabies-labeled neurons found not only ipsilaterally to LRN and FL injection but also in the contralateral spinal cord in Rexed's lamina 8 (Figures 3B–3D). In the longitudinal dimension, we observed a higher density of neurons at rostral spinal levels but a continuing presence of these neurons to anterior thoracic spinal levels (Figure 3C). Thus, dually connected neurons are not only restricted to cervical levels C3 to C4. Moreover, in the transverse dimension, the observed pattern was reminiscent of the overall FL-premotor distribution profile in the spinal cord (Figure 3D) (Stepien et al., 2010), including the specific locations of ipsi- and contralateral populations.

In summary, these findings demonstrate that propriospinal neurons with projections to the LRN and connections to FL-motor neurons in the mouse distribute across many spinal segments and locate both ipsi- and contralaterally in an overall pattern resembling the FL-premotor distribution. Thus, the LRN appears to sample the activity of a diverse set of premotor interneurons connecting to FL motor neurons as defined by position in the spinal cord. These observations raise the important question of whether and how this diversity of LRN-projecting neurons by spinal location is matched at the level of neuronal subpopulations with distinct genetic identity.

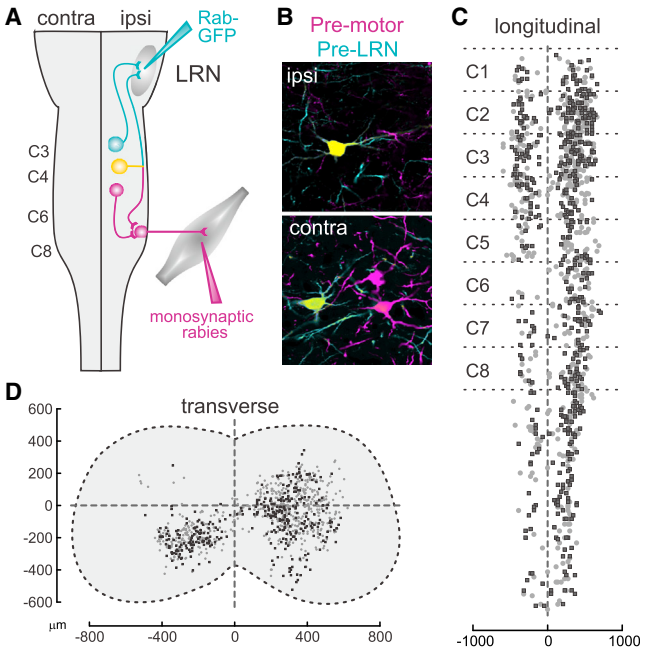

Figure 3. Source of Premotor Neurons to LRN Is Distributed across Spinal Cord

(A) Scheme of experimental setup displaying retrograde infection of FL LMC motor neurons from FL muscles by coinjection of Rab-mCherry and AAV-G protein to label FL-premotor neurons and injection of Rab-GFP into the LRN to retrogradely label neurons with projections to the LRN.

(B) Examples of neurons with dual-rabies infection ipsilateral (top) and contralateral (bottom) to limb-muscle injection.

(C) Longitudinal position of neurons with dual projections along the cervical and thoracic spinal cord (two representative reconstructions: black square and gray round symbols). Cervical spinal levels indicated, and scale depicts μm.

(D) Transverse position analysis of neurons with dual projections; compiled projection along spinal levels analyzed (see C). Scale depicts μm.

See also Figure S3.

Distinct LRN Termination Zones by Spinal Location and Neurotransmitter Fate

To directly address these questions, we next set up a strategy to visualize axon terminals in the LRN derived from spinal neurons of different origin. We made use of unilateral intraspinal injections of double-inverted-orientation-LoxP-flanked AAVs (AAV-flex) conditionally expressing a synaptophysin-GFP fusion protein (AAV-flex-SynGFP) upon Cre recombination, confined to either cervical (C4–C7) or lumbar (L1–L4) levels (Figures 4A and S4). To distinguish input to the LRN derived from excitatory (vGlut2^{ON}) and inhibitory (vGAT^{ON}) spinal neurons, we performed unilateral spinal coinjections of AAV-flex-SynGFP and AAV-flex-H2BGFP into either *vGlut2*^{Cre} or *vGAT*^{Cre} knockin mice (Vong et al., 2011) (Figures 4A and S4). This strategy allows separate mapping of excitatory or inhibitory ascending input from ipsi- or contralateral cervical and lumbar spinal cord to the LRN, independent of premotor character.

We found that unilateral cervical injections of AAV-flex-SynGFP into *vGlut2*^{Cre} or *vGAT*^{Cre} mice resulted in pronounced marking of axonal terminals in the entire core LRN ipsilateral to injection (Figure 4B), corresponding to the domain also marked by FL-premotor terminals. Within this domain, cervical-derived excitatory and inhibitory synapses were found at approximately equal density (Figure 4B). Combined marking of cervical neurons by intraspinal injection and FL-premotor neurons by monosynaptic rabies tools revealed the existence of both vGlut2^{ON} and vGAT^{ON} premotor terminals in the FL-dominated core area of the LRN (Figures 4E–4G), supporting their coincidence within this domain. Contralateral to spinal injection, only a low density of synaptic terminals compared to ipsilateral density was detected in the LRN of *vGlut2*^{Cre} and *vGAT*^{Cre} mice (Figure 4B). These sparse terminals were located in the ipsilateral FL-dominated LRN domain but also extended more medially into a domain not targeted by ipsilateral neurons (Figure 4B). In summary, the major targeting domain for cervical spinal neurons within the LRN is an ipsilateral, centrally located FL-premotor LRN domain, within which no preferential distribution of excitatory and inhibitory terminals can be observed (Figure 4D).

Upon lumbar spinal cord injections, we detected almost exclusively glutamatergic terminals at high densities in the LRN with targeting domains spatially distinct from the FL population (Figure 4C). Both ipsi- and contralateral vGlut2^{ON} terminals were restricted to a highly confined area ventrally to the one targeted by cervical neurons (Figures 4C and 4D). Contralateral lumbar interneurons targeted the domain just ventral to the ipsilateral cervical territory, and ipsilateral lumbar interneurons targeted the adjacent more lateral domain (Figures 4C and 4D). The very sparse contralateral vGAT^{ON} terminals were confined to the most medial and ventral corner of the LRN (Figure 4C).

Together, these findings reveal the existence of a spatial map within the LRN. Input from cervical and lumbar segments of ipsi- and contralateral sides is confined to distinct territories within the LRN (Figure 4D). The LRN area targeted by ipsilateral FL spinal input represented the most dominant input to the LRN from the spinal cord. It exhibits shared occupation by excitatory and inhibitory terminals and coincides with the premotor domain. These findings raise the question of whether within this domain of intermingled terminals, spatial input segregation might occur according to the identity of functionally distinct spinal subpopulations.

Distinct LRN Termination Zones by Progenitor-Domain Identity

Our analysis of bifurcating spinal neurons with projections to the LRN uncovered a much more widespread population of neurons than previously anticipated. To determine whether these fractionate into functionally distinct spinal populations as defined by developmental origin from different progenitor domains, we concentrated specifically on the cervical spinal cord. This focus would allow us to address diversity of origin and to establish a possible correlation between genetic identity and axonal targeting within an LRN subdomain. Most genes expressed specifically in identified spinal progenitor domains or in early postmitotic neuronal subpopulations are downregulated rapidly at embryonic stages (Figure 5A) (Alaynick et al., 2011), preventing the direct use of Cre mouse lines at postnatal stages to mark neurons derived from these progenitor domains by intraspinal viral injections. To overcome this limitation, we implemented a

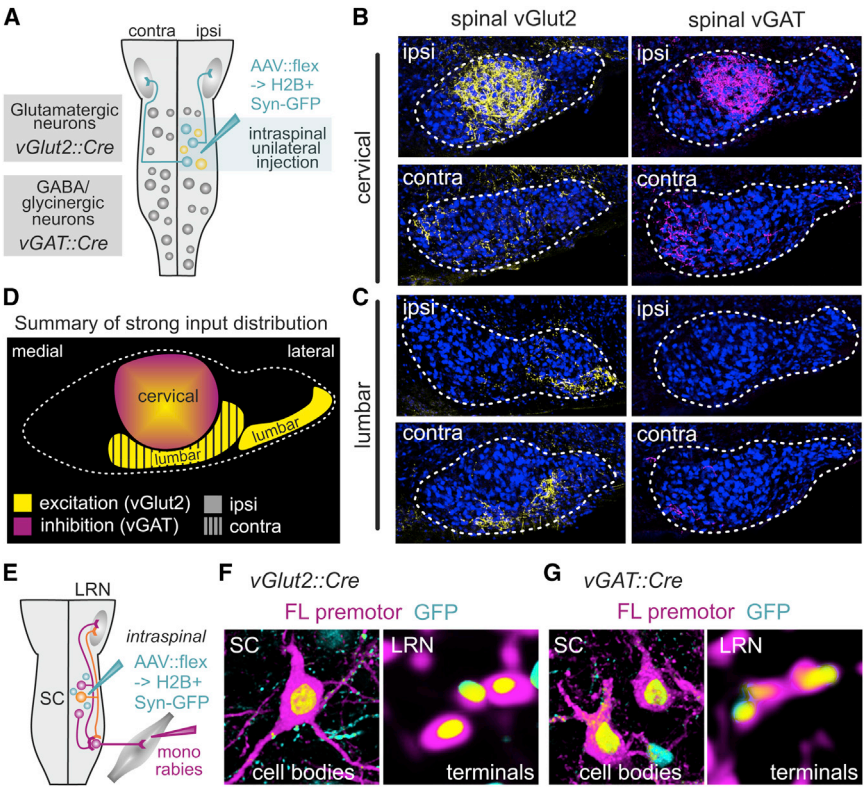

Figure 4. Differential Spinal Ascending Pathways by Neurotransmitter and Spinal Origin

(A) Scheme of experimental setup for unilateral intraspinal injections of flexed AAVs (H2B and SynGFP) into *vGlut2*^{Cre} mice to mark glutamatergic spinal neurons and *vGAT*^{Cre} mice to mark GABA and glycinergic spinal neurons.

(B and C) Coronal LRN sections are shown displaying NeuN and SynGFP on sides ipsilateral (top) or contralateral (bottom) to spinal injections. Dashed lines delineate outer LRN border. Cervical (B) and lumbar (C) injections are shown separately (medial LRN to the left, lateral LRN to the right in all panels).

(D) Summary diagram of results displayed in this figure (only strong anatomical input depicted in model). Note fractionation of the LRN into different territories by spinal origin and neurotransmitter identity of neurons.

(E) Scheme of experimental setup identical to (A) but with broad unilateral monosynaptic rabies injection into FL muscles.

(F and G) FL-premotor neurons coinfected by flexed AAV (H2B and SynGFP) at the level of the spinal cord (left) and resulting high-resolution terminals in the LRN (right) are shown to confirm premotor status of ascending vGlut2 (F) and vGAT (G) positive neurons.

See also Figure S4.

novel intersectional mouse transgenic system combined with intraspinal viral injections for our experiments (Figures 5B–5D).

Our approach involved the generation of a new transgenic mouse line to conditionally express Flp recombinase from the pan-neuronal *Tau* locus (*Tau*$^{lox\text{-}STOP\text{-}lox\text{-}FLP\text{-}INLA}$ or *Tau*$^{lsl\text{-}FLP}$ mice; Figure 5B), a locus previously used successfully to express transgenes in specific neuronal subpopulations (Hippenmeyer et al., 2005; Stepien et al., 2010; Tripodi et al., 2011). We found that intersectional breeding between Cre lines with transient embryonic expression and *Tau*$^{lsl\text{-}FLP}$ mice led to permanent nls-LacZ and Flp recombinase expression in derivative neurons (Figures 5E and S5). Intraspinal injection of double-inverted-orientation-FRT-flanked AAVs (AAV-frted) to conditionally express GFP in these mice at postnatal stages can thus be used to visualize mature neuronal subpopulations (Figures 5D, 5E, and S5) and their axon terminals, despite only transient expression of Cre recombinase during development at embryonic stages (Figures 5A and 5C).

Using this approach (Figure 6A), we first selected two Cre lines that mark the neuronal descendants of single progenitor domains giving rise to excitatory vGlut2^{ON} spinal subpopulations. These were the ventrally derived V2 (*Lhx3*^{Cre}; mostly V2a—see Experimental Procedures) and the dorsally derived dI3 (*Isl1*^{Cre}) populations (Figure 6B). Unilateral intraspinal injection of AAV-frted-GFP into *Lhx3*^{Cre}/*Tau*$^{lsl\text{-}FLP}$ or *Isl1*^{Cre}/*Tau*$^{lsl\text{-}FLP}$ mice marked selectively respective neuronal subpopulations at the level of the spinal cord (Figure 5E). Analysis of SynGFP fluorescence in the LRN in these mice demonstrated that both V2 and dI3 subpopulations establish ipsilateral termination zones (Figure 6C), but whereas Lhx3-SynGFP synapses are found ventro-medially, Isl1-SynGFP terminals preferentially target a lateral domain (Figure 6C).

We next conducted a similar analysis with a mouse line expressing Cre recombinase in a single progenitor domain giving rise to inhibitory spinal neurons, the ventrally derived V1 population (*En1*^{Cre}) (Figures 6B and S5). We found that En1-SynGFP-tagged neurons establish dense and widespread axonal terminals within the FL territory of the LRN ipsilateral to spinal injection, overlapping with and unlike the confined termination zones observed for V2 and dI3 neurons in the LRN (Figure 6C).

To assess whether spinal neurons projecting to contralateral LRN territory can be distinguished by their progenitor-domain origin as well, we chose the V3 population known to establish excitatory commissural projections at the level of the spinal cord (Zhang et al., 2008) (Figure 6B). Intraspinal injection of AAV-frted-GFP into *Sim1*^{Cre}/*Tau*$^{lsl\text{-}FLP}$ mice revealed that these neurons establish a selective termination zone in the contralateral LRN located in the ventral FL-targeted LRN territory (Figure 6C), providing further evidence for specific targeting of functionally distinct spinal populations to LRN subdomains.

To determine whether ascending terminals of different progenitor-domain origin encompass FL-premotor populations, we combined intraspinal AAV injections with monosynaptic rabies injections into FL muscles (Figures 6A and 6D). We subsequently assessed whether we could detect synaptic terminals in the LRN, marked both by the spinally expressed SynGFP tag and by FL-premotor rabies virus fluorescence. In experiments carried out in *Lhx3*^{Cre}, *Isl1*^{Cre}, *En1*^{Cre}, and *Sim1*^{Cre} genetic

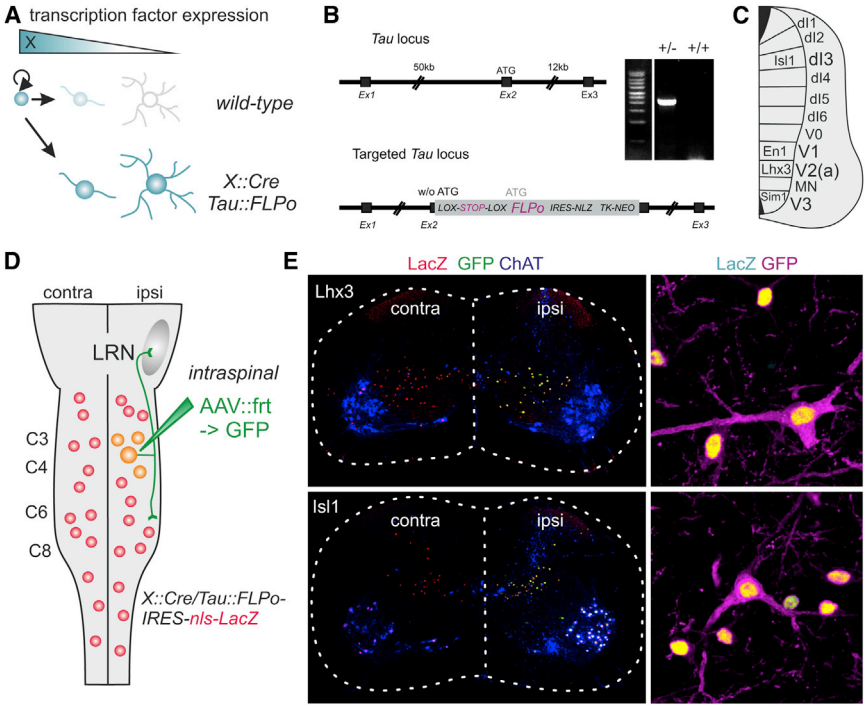

Figure 5. Intersectional Genetic Tool for Targeting Developmentally Marked Subpopulations

(A) Scheme depicts frequently observed downregulation of transcription factor (X) expression by genetically defined progenitors or early postmitotic neurons. Conditional activation through Cre-mediated intersectional breeding between *X::Cre* and *Tau::FLPo-IRES-nls-LacZ* mice allows permanent marking of corresponding neurons by NLS-LacZ and FLP recombinase expression.

(B) Generation of knockin mouse for conditional expression of FLP recombinase from the pan-neuronal *Tau* locus, by integration into exon 2 of the locus. PCR to confirm positive recombination event is displayed next to DNA ladder on gel.

(C) Scheme displaying subdivision of embryonic spinal cord in 11 distinct progenitor domains and emergent neuronal subpopulations. Transcription factor code is displayed for populations studied here (dI3: Isl1; V1: En1; V2(a): Lhx3; V3: Sim1).

(D) Scheme of experimental setup for unilateral intraspinal injections of FRT-flanked AAVs into *X::Cre/Tau::FLPo-IRES-nls-LacZ* mice to mark synapses of neurons derived from defined spinal progenitor domain.

(E) Representative pictures of unilateral spinal injections of FRT-flanked AAVs into $Lhx3^{Cre}$ and $Isl1^{Cre}$ mice crossed with *Tau::FLPo-IRES-nls-LacZ* mice (high-resolution example neurons depicted to the right).

See also Figure S5.

backgrounds, we found double-labeled terminals for all four conditions (Figure 6D). In summary, these findings establish that spinal neurons of different single progenitor-domain origin establish distinct axonal terminations in the LRN, including FL-premotor populations. However, excitatory spinal subpopulations marked by these criteria target more confined LRN subdomains than inhibitory counterparts.

LRN-Projecting Genetic Subpopulations Are Spatially Confined in the Spinal Cord

The striking spatial organization of synaptic terminals in the LRN derived from distinct spinal subpopulations raised the complementary question of whether also at the level of the spinal cord, LRN-projecting neurons are found in spatially restricted domains, a finding that could have important functional consequences.

We first assayed the spatial distribution of neurons derived from different progenitor domains in the mature cervical spinal cord. For this purpose, we used intersectional breeding between $Lhx3^{Cre}$, $Isl1^{Cre}$, $En1^{Cre}$, and $Sim1^{Cre}$ with $Tau^{Isl\text{-}FLP}$ mouse lines. This strategy permanently marks corresponding neurons by LacZ expression ($Lhx3^{LacZ}$, $Isl1^{LacZ}$, $En1^{LacZ}$, $Sim1^{LacZ}$), allowing reconstruction of cell-body position of these populations in the spinal cord. We found that $Lhx3^{LacZ}$ and $Isl1^{LacZ}$ neurons distributed to largely nonoverlapping spatial territories, with V2-derived neurons found in a more ventral position than dI3 neurons (Figures 7A and 7B). $En1^{LacZ}$ neurons distributed more broadly along the dorsoventral axis but overall were located in closer proximity to LMC motor neurons than $Lhx3^{LacZ}$ or $Isl1^{LacZ}$ neurons, whereas $Sim1^{LacZ}$ neurons were located in an extreme medial and ventral position (Figures 7A and 7B). Quantitative analysis further demonstrated that V1 neurons were much more abundant than V2, dI3, or V3 neurons (Figure 7C), suggesting that perhaps V1 neurons fail to demonstrate LRN-targeting specificity due to further division into distinct subpopulations beyond single-progenitor-domain origin.

The observed spatial segregation of neurons derived from individual progenitor domains at the level of the spinal cord raises the question of whether LRN-projecting premotor neurons of a given progenitor-domain identity follow the same organizational principle. To address this issue, we chose V2 neurons as an exemplary population. We crossed $Lhx3^{Cre}$ and $Tau^{Isl\text{-}FLP}$ mice to visualize corresponding spinal neurons and carried out the dual-rabies labeling approach marking spinal neurons with connections to FL-motor neurons and projections to the LRN (Figure 7D). We found that $LacZ^{ON}$ neurons made up approximately 30% of the dual-rabies-labeled ipsilateral population, and that these neurons were confined to a medial domain in the intermediate spinal cord (Figures 7E and 7F). The distribution of these neurons was similar to the distribution of the overall $Lhx3^{ON}$ premotor population (Figure 7F), suggesting that dual-connecting premotor-LRN neurons make up a subset of the general population, transmitting an excerpt of ongoing activity of premotor neurons to the LRN.

In summary, these findings provide evidence that developmental progenitor-domain origin in the spinal cord prefigures stereotype and spatially confined spinal-settling positions of derivative neuronal subpopulations, as well as the establishment

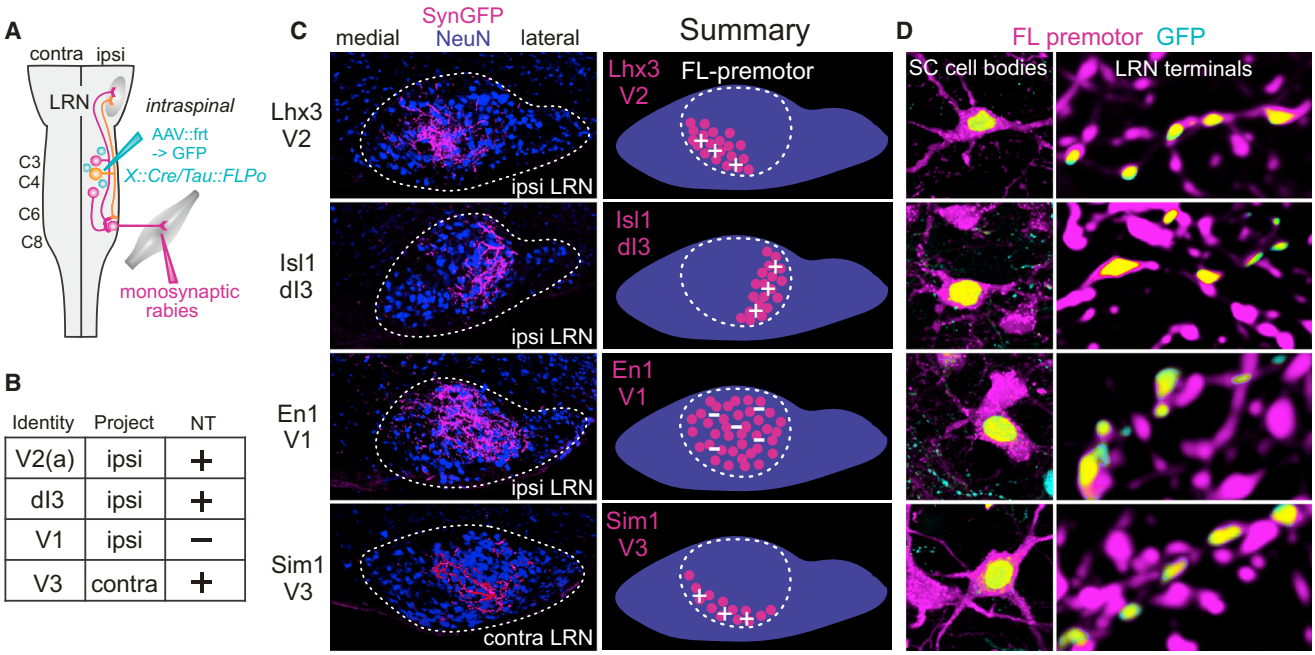

Figure 6. Genetically Identified Spinal Populations Exhibit Distinct LRN Termination Zones

(A) Experimental setup with unilateral intraspinal injection of FRT-flanked AAVs (C) and with unilateral monosynaptic rabies injection into FL muscles (D).

(B) Summary diagram of neuronal identity, projection target, and neurotransmitter (NT) identity (+: vGlut2^{ON}; –: vGAT^{ON}) of studied spinal subpopulations.

(C) Visualization of axonal terminals (SynGFP) derived from cervical spinal neuron subpopulations marked by progenitor-domain origin (Lhx3, Isl1, En1, and Sim1) on coronal caudal LRN sections. Left panels depict LRN ipsilateral to spinal injection for Lhx3, Isl1, and En1 and contralateral LRN for Sim1 experiments. Right panels depict summary diagram of observed synaptic inputs to different domains of the FL-premotor LRN territory by different spinal populations.

(D) Dual-labeling experiments with monosynaptic rabies viruses to determine premotor status of labeled spinal neurons (left; H2B-GFP marked) and high-resolution synaptic terminals in the LRN (right; SynGFP marked). For Sim1 experiments, monosynaptic rabies viruses were injected contralaterally to spinal injections.

of specific ascending axonal targeting domains to the LRN in the brainstem.

DISCUSSION

Motor-circuit collaterals are the internal neuronal substrate for corrective signaling during the execution of motor tasks. Here we unravel the synaptic organization and origin of such collaterals carrying motor-related information from the spinal cord to supraspinal centers. We demonstrate the existence of a precisely organized connectivity matrix between diverse and genetically distinct spinal populations and stereotypical LRN subdomains. We discuss our findings in the context of organizational and functional properties of ascending spinal signaling systems and their role in the control of motor behavior.

Ascending Signals to the LRN Established by Distinct Spinal Subpopulations

Our work demonstrates that FL-premotor LRN-projecting PNs fractionate into many diverse spinal populations based on genetic criteria. In the past, "C3-C4 PNs" were thought of as a singular neuronal population located at segmental levels C3 to C4 in the ipsilateral spinal cord, only divided into an excitatory and inhibitory population (Alstermark and Isa, 2012; Alstermark et al., 1984, 2007). In fact, evidence for the existence of an inhibitory subpopulation has been sparse due to the inherent difficulty to separate excitatory from inhibitory axons by antidromic stimulation experiments from the LRN (Alstermark et al., 1984).

We show that the overall distribution of these dual-connection neurons in the spinal cord is much broader than just C3 to C4 spinal levels, with cell bodies positioned throughout cervical and extending into the thoracic spinal cord. This discovery was made possible by the combination of novel mouse genetic tools and connectivity-based circuit-mapping approaches of high sensitivity, as compared to electrophysiological recordings in the in vivo spinal cord in the past. These novel methods also enabled us to uncover that premotor neurons in the spinal cord giving rise to LRN-projecting axons encompass both ipsi- and contralateral populations. We note that the overall density of FL-premotor LRN projection neurons is higher at rostral than at caudal spinal levels, as well as on the ipsi- rather than contralateral spinal side, providing a possible explanation for why it was too challenging to detect the overall distribution of these neurons with electrophysiological techniques.

Functional Implications of Genetically Diverse Spinal Channels to the LRN

Our results demonstrate that subpopulations of cervical spinal neurons with distinct genetic identities by developmental ontogeny establish axons terminating in stereotypic territories

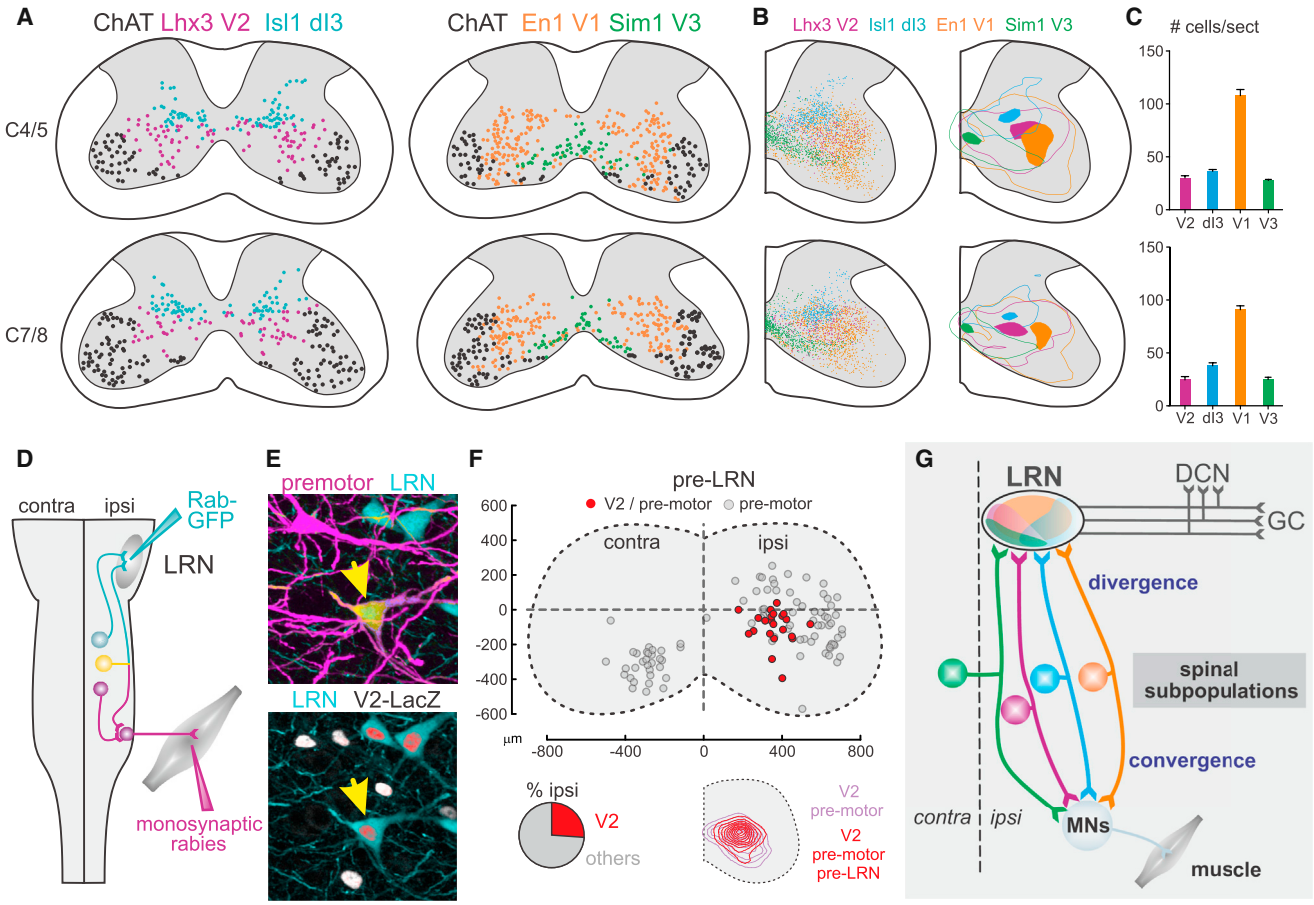

Figure 7. Spinal Location of LRN-Projecting Subpopulation Reveals Spatial Segregation

(A) Spatial distribution of LacZ^{ON} spinal neurons in *Lhx3::Cre*, *Isl1::Cre*, *En1::Cre*, and *Sim1::Cre* mice crossed with *Tau::FLPo-IRES-nls-LacZ* mice. Representative sections at C4/5 and C7/8 spinal levels are shown, and LMC motor neurons are shown in black.

(B and C) Overlay of scatter plots of neuronal position (left), isolines of neuronal distributions (middle; filled territory: 20%; outer line: 90% of neurons around highest density), and numbers of neurons per 40 µm section (average over 12 sections; n = 3 mice; ± SEM) for data shown in (A).

(D) Scheme of experimental setup in analogy to Figure 3A. Assay is used to determine position of neurons with projections to the LRN and connections to FL-motor neurons in the spinal cord.

(E) Representative pictures of experiments carried out in *Lhx3::Cre* mice crossed with *Tau::FLPo-IRES-nls-LacZ* mice to depict LacZ, LRN projection, and FL-premotor status. Yellow arrow depicts exemplary triple-positive neuron.

(F) Positional analysis of pre-LRN/premotor and V2/pre-LRN/premotor neurons shown on transverse projection. Bottom shows that V2 neurons make up approximately 30% of ipsilateral pre-LRN/premotor population (left), and distribution analysis depicts that the two populations are not distinctly localized (right).

(G) Summary diagram depicting main findings. Spinal neuron subpopulations diversify by developmental genetic identity and spatial distribution in the spinal cord. Both ipsi- and contralaterally located subpopulations establish dual projections to cervical motor neurons and the LRN in the brainstem. Subpopulation input convergences on spinal motor neurons and divergences to different territories in the LRN. LRN neurons project to deep cerebellar nuclei (DCN) and cerebellar granule cells (GC).

within the LRN FL domain (Figure 7G). These neuronal populations also exhibit distinct spatial distributions in the spinal cord. Together, our findings raise the question of possible implications of such highly organized efference copy-signaling arrangements for the function of these circuits in controlling motor behavior.

It is well established that neurons derived from different progenitor domains exhibit distinct intraspinal functions (reviewed by Alaynick et al., 2011; Arber, 2012; Goulding, 2009; Grillner and Jessell, 2009; Kiehn, 2011). For example, a recent study demonstrated the importance of dI3 interneurons in the execution of grasping behavior in mice (Bui et al., 2013). Thus, genetic programs initiated at early developmental stages in specific spinal subpopulations prefigure their functional properties in the adult spinal cord, including the control of neuronal settling position as well as synaptic input and output patterns. Our study demonstrates that subpopulations with distinct intraspinal functions transmit these differences to the brainstem in highly organized ascending information channels to different neurons in the LRN. This signaling setup has the strong advantage of coincident and faithful transfer of premotor information to the LRN without synaptic intermediary. Most importantly, whereas an individual motor neuron pool unlikely discriminates the identity of a premotor population providing synaptic input, our findings

demonstrate that much in contrast, the ascending axonal branches of these same premotor neurons segregate and combine according to their spinal subpopulation identity. As an important functional consequence, synaptic information convergent at the motor-pool level diverges in the LRN (Figure 7G).

We found that, in addition to the functional diversity of neuronal subpopulations revealed through genetic entry points, the spatial distribution of dual-connection neurons is highly reminiscent of the overall FL-premotor interneuron distribution (Stepien et al., 2010). This observation lends further support to a model in which ascending LRN-signaling pathways are composed of many functionally distinct premotor spinal populations, transmitting an excerpt of perhaps even almost all ongoing motor-output-related spinal activity to the LRN. It also provides an explanation for why synaptic terminals of premotor neurons connected to one motor pool as a whole do not exhibit a discriminatory LRN axonal-targeting pattern. Instead, each FL-premotor population linked to an individual motor neuron pool fractionates into many genetically and functionally distinct subpopulations following separate rules for LRN axon targeting, resulting in an overall targeting map based on spinal subpopulation identity.

LRN Information in the Cerebellar Loop and Influence on Descending Pathways

Our findings on the exquisite synaptic organization of spinal input to the LRN raise the question of the further transmission of this information through the cerebellar loop. The LRN is composed exclusively of projection neurons to the cerebellum and thought to be a pure integration nucleus with no local computation. LRN mossy fibers project to cerebellar granule cells but at the same time give off a collateral to deep cerebellar nuclei (Shinoda et al., 2000; Wu et al., 1999). Granule cells transmit information in highly divergent connectivity patterns to Purkinje cells, which in turn provide the output of the cerebellum to deep cerebellar nuclei (Arshavsky et al., 1986; Ito, 2006).

Previous work trying to disentangle the projection specificity of LRN neurons to cerebellar target lobules provides evidence that a dorsally located "region A" within the LRN may exhibit preferential ipsilateral cerebellar projections, whereas a ventral "region B" projects bilaterally (Clendenin et al., 1974a). Although crude due to technical limitations, in light of the differential spatial targeting of the LRN by distinct spinal populations shown here, these findings might imply that transmission of the separate spinal channels may be carried on by the LRN mossy-fiber system to deep cerebellar nuclei and the cerebellar cortex. In this context, it will be interesting to assess how deep cerebellar nuclei combine excitatory collateral LRN mossy fiber and inhibitory Purkinje cell information to determine how the spatial map observed in the LRN is transformed at this level. Deep cerebellar nuclei exert profound synaptic influence on several descending brainstem nuclei (reticulo-spinal; vestibular nucleus, red nucleus) (Arshavsky et al., 1986; Ito, 2006; Orlovsky et al., 1999), which deliver this information updated through the cerebellar loop to the spinal cord. It can be expected that the spinal cord-LRN-cerebellum-deep cerebellar nuclei loop is closed and provides means to update descending pathways in a timely fashion with ongoing activity from the spinal cord. Indeed, a subset of spinal V2a neurons was found by electrophysiology to provide coincident monosynaptic input to FL-motor neurons and LRN neurons and to influence goal-directed reaching behavior in mice (Azim et al., 2014).

General Principles of Ascending Signaling-System Organization

LRN-projecting neurons in the spinal cord also exhibit striking organization beyond the cervical populations. Cervical and lumbar spinal neurons establish separate terminations within the LRN, a finding in line with previous electrophysiological recordings and anatomical experiments (Brodal, 1949; Clendenin et al., 1974b; Ekerot, 1990; Künzle, 1973). We find that ipsilateral cervical terminals reside immediately dorsal to contralateral lumbar terminals within the LRN. This arrangement is intriguing considering the normal rodent two-beat gait exhibited during locomotion, with coincident activity of diagonal limbs and parallel action patterns of ipsilateral FL and contralateral HL. Nevertheless, however, the HL-dominated ventral territory in the LRN does not receive input from lumbar premotor neurons, raising the question of whether alternative channels for transmission of HL-premotor information exist.

Electrophysiological studies in the cat lumbar spinal cord demonstrate that inhibitory premotor interneurons connect to neurons projecting through the ventral spinocerebellar tract (VSCT) (Jankowska and Hammar, 2013; Jankowska et al., 2010; Lundberg, 1971). VSCT neurons establish direct mossy-fiber projections from the lumbar spinal cord to the cerebellum and are proposed to be functional equivalents of LRN neurons monitoring spinal cord intrinsic activity in the lumbar spinal cord (Orlovsky et al., 1999; Oscarsson, 1965). Recent anatomical work retrogradely labeling lumbar neurons with projections to the cerebellum demonstrates that VSCT neurons are targeted predominantly by inhibitory neurons (Shakya Shrestha et al., 2012; Shrestha et al., 2012). Whether and how synaptic targeting specificity also exists for input to VSCT neurons in a manner similar to that for the LRN will be important questions to address in the future. The overall picture that emerges is one of a binary transmission system for spinal information related to FL or HL motor output to the cerebellum. The most universal one with a dedicated FL-premotor domain is the LRN, and a second one is embedded within the lumbar spinal cord as VSCT neurons transmitting more local lumbar events to the cerebellum.

In summary, our findings provide evidence for precise organization of ascending spinal information to the brainstem, encompassing many functionally distinct spinal subpopulations, which can be divided by site of residence in the spinal cord, developmental origin, and neurotransmitter fate. We provide insight into the genetic complexity of the spinal efference copy-signaling system, lending support to the notion that listening attentively to ongoing activity of the spinal cord at supraspinal levels provides an important prerequisite for accuracy in motor control.

EXPERIMENTAL PROCEDURES

For the generation of *Tau*$^{lox\text{-}STOP\text{-}lox\text{-}FLP\text{-}INLA}$ mice, a *lox-STOP-lox-FLPo-IRES-Nls-LacZ-pA* targeting cassette was integrated into exon 2 of the *Tau* locus using a strategy described previously (Hippenmeyer et al., 2005). Additional

mouse lines used in this study are described in the Extended Experimental Procedures. Rabies virus experiments with monosynaptic restriction to label premotor neurons were carried out as described previously for both production and injection (Stepien et al., 2010; Tripodi et al., 2011). Additional description on production and injections of viruses, antibodies, imaging, and statistical analysis can be found in the accompanying Extended Experimental Procedures.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures and five figures and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2013.12.014.

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